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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Targeted radiation therapy for breast cancer is a rational approach, however; there are problems associated with low receptor expression on tumors and bone marrow toxicity due to the long serum half-life of antibodies used to target the radiation. An adenoviral vector encoding the human somatostatin receptor subtype 2 (AdSSTr2) has been produced. The MDA-MB-468 and BT-474 human breast cancer cells were infected with AdSSTr2 and harvested 48 h later for membrane preparations. Membrane SSTr2 expression was determined by a competitive binding assay using iodine-125 (I-125)-labeled somatostatin. Localization of Tc-99m-P2045 (a high affinity somatostatin analogue) in mice bearing subcutaneous tumors was evaluated by injecting AdSSTr2 i.v. followed by an i.v. injection of Tc-99m-P2045 48 h later. Localization of Tc-99m-P2045 was determined by counting tissues in a gamma counter. These studies provide the proof-of-principle that this novel paradigm can be used to treat and detect breast cancer.

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Introduction

Targeted radiation therapy for breast cancer is a rational approach, however; there are problems associated with low receptor expression on tumors and bone marrow toxicity due to the long serum half-life of antibodies used to target the radiation. A novel method to overcome these problems was investigated. An adenoviral vector encoding the human somatostatin receptor subtype 2 (AdSSTr2) was produced. The MDA-MB-468 and BT-474 human breast cancer cells were infected with AdSSTr2 and harvested 48 h later for membrane preparations. Membrane SSTr2 expression will be determined by a competitive binding assay using iodine-125 (I-125)-labeled somatostatin. Localization of technetium-99m (Tc-99m)-P2045 (a high affinity somatostatin analogue) in mice bearing subcutaneous MDA-MB-468 tumors was evaluated by injecting AdSSTr2 i.v. followed by an i.v. injection of Tc-99m-P2045 48 h later. Localization of Tc-99m-P2045 was determined by counting tissues in a gamma counter. Therapy studies were to be conducted using AdSSTr2 followed by therapeutic doses of i.v. administered Y-90-SMT 487, however, time did not allow the completion of these studies. Overall, these studies provided the proof-of-principle that this novel paradigm can be used to treat and detect breast cancer.

Body

This award was a Breast Concept award and did not require a Statement of Work like other DOD grants. The aims of the proposal were to: 1.) use AdSSTr2 to infect MDA-MB-468 and BT-474 human breast cancer cells and evaluate the expression of SSTr2 by a competitive binding assay, 2.) evaluate SSTr2 expression in a mouse model of human breast cancer after injection of AdSSTr2 and injection of ^{99m}Tc-P2045 48 h later by animal biodistribution, and 3.) perform therapy studies in this same model using the therapeutic peptide ^{90}Y-SMT 487.

Methods

Cells. The MDA-MB-468 and BT-474 human breast cancer cells and the 293 human transformed primary embryonal kidney cells were obtained from the American Type Culture Collection (Rockville, MD). The MDA-MB-468 cells were maintained in DMEM containing 10% fetal calf serum (FCS) and 1 mM L-glutamine. The BT-474 cells were maintained in RPMI

containing 10% FCS, 1 mM L-glutamine, and 0.01 mg/ml bovine insulin. The 293 cells were maintained in EMEM containing 10% FCS, 1% non-essential amino acids and 1% sodium pyruvate. All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Construction of AdCMVhSSTr2. A recombinant adenovirus encoding the human somatostatin receptor subtype 2 cDNA was prepared using standard techniques described by Graham and Prevec (1). This is similar to the method described previously for the construction of the adenovirus encoding the murine gastrin releasing peptide receptor (2). Briefly, a cDNA fragment containing the hSSTr2 gene (obtained from the American Type Culture Collection) was subcloned into the pACCMVpLpARS(+) adenoviral shuttle vector (provided by R.Gerard, Katholieke Universiteit Leuven, Leuven, Belgium). This shuttle plasmid (pAC-hSSTr2) was cotransfected into the E1A transcomplementing cell line 293 with the adenoviral packaging plasmid pJM17 (provided by F. Graham, McMaster University, Hamilton, Ontario, Canada) using the DOTAP (Life Technologies, Inc., Gaithersburg, MD) cationic liposome vector. The recombinant adenovirus was plaque purified and validated by PCR. The AdSSTr2 was titered within the 293 cell line using plaque assay techniques for direct determination of viral pfu.

Competitive Binding Analyses. The induction of SSTr2 in human breast cancer cell lines was evaluated using a radiolabeled peptide binding assay to cell membrane preparations of cells that had been infected with AdSSTr2. The cells were seeded such that they were ~80% confluent at the time they were infected with AdSSTr2 and then harvested for membrane preparations 2 days after adenoviral infection. The AdSSTr2 (50 pfu per cell) was added to cells in Optimem® (Gibco-BRL, Grand Island, NY) and incubated at 37°C in 5% CO_2 for 2 h. The cells were then supplemented with complete media and incubated an additional 48 h at 37°C. Cell membranes were then prepared from the infected and uninfected cells using a protocol similar to that previously described (3-6). Briefly, the cells were washed with phosphate buffered saline, scraped from the flask, and centrifuged at 90 x g for 5 min at 4°C. The pellet was resuspended in cold lysis buffer (10 mM Tris-HCl, 2 mM EDTA, 2 mM $MgCl_2$, pH 7.2) containing 0.5 mM phenylmethylsulfonyl fluoride and incubated on ice for 15 min. The mixture was vortexed, centrifuged at 600 x g for 15 min at 4°C, and the supernatant removed and stored on ice. An additional lysis step was performed on the pellet and the two supernatants were combined. The supernatant was centrifuged at 28,000 x g for 30 min at 4°C, the resulting supernatant discarded, and the pellet resuspended in 250 mM sucrose, 20 mM glycylglycine, and

1 mM $MgCl_2$. A BioRad (Hercules, CA) protein assay was performed to determine the protein concentration and the samples aliquoted and stored at -80°C.

For the binding assays, the membrane preparations were thawed and diluted in buffer (10 mM HEPES, 5 mM $MgCl_2$, 1 mM EDTA and 0.1% bovine serum albumin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 0.5 μg/ml aprotinin, and 200 μg/ml bacitracin, pH 7.4) to 25 μg per sample. Individual samples were added to Multiscreen Durapore filtration plates (type FB, 1.0 μm borosilicate glass fiber over 1.2 μm Durapore membrane; Millipore, Bedford, MA) and washed with buffer (10 mM HEPES, 5 mM $MgCl_2$, 1 mM EDTA and 0.1% bovine serum albumin, ph 7.4). One hundred μl [^{125}I]-Tyr^1-somatostatin (~10,000 cpm; specific activity = 1400-2200 Ci/mmol; DuPont/NEN® Research Products, Boston, MA) was added to each well along with various concentrations of Tyr^1-somatostatin (0 to 450 nM) as an inhibitors and incubated for 90 min at room temperature. The samples were washed twice with ice-cold buffer, the filters allowed to dry, and the individual wells punched out and counted in a gamma counter. The binding constants (K_d) and receptor density (B_{max}) was calculated using the GraphPad Prism software (San Diego, CA).

Animal Biodistribution Studies. Biodistribution studies were performed in groups of 3-5 athymic nude mice (National Cancer Institute Frederick Research Laboratory, Frederick, MD) bearing subcutaneous human breast tumors. Mice were implanted subcutaneously with 1 x 10^7 MDA-MB-468 cells in the rear flank mixed 1:1 with matrigel. Three weeks later the mice were injected i.v. with 1 x 10^9 pfu of AdSSTr2 or a control adenovirus. Two days later the mice were injected i.v. with 10 μCi of ^{99m}Tc-P2045 and the animals sacrificed 4 h later. Blood, lungs, liver, kidney, muscle, pancreas, and tumor were harvested, weighed and counted in a gamma counter. The data are represented as the % injected dose (ID) per gram of tissue.

Results

A representative competitive binding assay is shown in **Figure 1**. This shows that both cell lines express a high level of SSTr2 after infection with AdSSTr2 at 50 pfu/cell. This assay was conducted in triplicate to determine the K_d and B_{max} values as shown in **Table 1**. This shows that [^{125}I]-Tyr^1-somatostatin bound with high affinity to both BT-474 cells (0.8 ± 0.4 nM) and MDA-MB-468 cells (16.2 ± 16.1 nM). Also, both cells lines expressed a high level of SSTr2,

but the MDA-MB-468 cells expressed more than the BT-474 cells (99,600 ± 77,000 fmol/mg vs. 6,700 ± 200 fmol/mg). Due to the greater expression of SSTr2 in MDA-MB-468 cells, these cells were evaluated in the nude mouse model.

The biodistribution results are shown in **Figure 2**. This shows that expression of SSTr2 is observed on tumor and other tissues after infection with AdSSTr2. This expression is greater than when mice were infected with a control adenovirus. The tumor uptake of ^{99m}Tc-P2045 increased from 1.3 ± 0.6 % ID/g after infection with the control adenovirus to 5.9 ± 2.0 % ID/g after infection with AdSSTr2. Similarly, the liver uptake of ^{99m}Tc-P2045 increased from 0.7 ± 0.2 % ID/g after infection with the control adenovirus to 36.1 ± 25.0 % ID/g after infection with AdSSTr2. This represents a > 50-fold increase in the uptake of ^{99m}Tc-P2045 in the liver. Other tissue showed a 2-5-fold increase in ^{99m}Tc-P2045 uptake after injection of AdSSTr2 when compared to controls. These studies demonstrate that AdSSTr2 can infect MDA-MB-468 cells after i.v. injection, which can accumulate ^{99m}Tc-P2045.

Key Research Accomplishments

- Demonstration that AdSSTr2 could be used to induce expression of SSTr2 on BT-474 and MDA-MB-468 human breast cancer cells.
- The expression of SSTr2 was greater on MDA-MB-468 cells than on BT-474 cells after AdSSTr2 expression.
- AdSSTr2 could induce expression of SSTr2 on subcutaneous MDA-MB-468 tumors as shown by uptake of ^{99m}Tc-P2045.

Reportable Outcomes

None, these results have not been written-up for an abstract or manuscript. It is likely that more data will need to be collected prior to submission of a manuscript.

Conclusions

In conclusion, these studies show that AdSSTr2 can be used to express SSTr2 in BT-474 and MDA-MB-468 human breast cancer cells. The expression of SSTr2 was greater in the MDA-MB-468 cells than in the BT-474 cells. It was also demonstrated that i.v. injection of AdSSTr2 could induce expression of SSTr2 in mice bearing s.c. MDA-MB-468 tumors. Direct intratumoral injection of AdSSTr2 should yield even greater tumor expression of SSTr2 and reduce SSTr2 expression in normal tissues. This will allow injection of a therapeutic radiolabeled peptide (^{90}Y-SMT 487) to treat the SSTr2-expressing tumors. More studies need to be performed to demonstrate this, however, this Breast Concept Award has allowed the initial proof of principle data to be generated.

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Appendices

Figure and Table Legend

Figures and Table

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Figure 1. Competitive inhibition curves of [^{125}I]-Tyr^{1}-somatostatin binding to BT-474 and MDA-MB-468 human breast cancer cell membrane preparations. Various concentrations of Tyr^{1}-somatostatin were used to inhibit ^{125}I-Tyr^{1}-somatostatin binding. Data are plotted as cpm bound vs. Log of the molar concentration of ^{125}I-Tyr^{1}-somatostatin. Each point is the mean of triplicate measurements ± standard deviation.

Table 1. Binding affinity (K_d) of [^{125}I]-Tyr^{1}-somatostatin to AdSSTr2-infected BT-474 and MDA-MB-468 cell membrane preparations andSSTr2 expression (B_{max}; fmol/mg).

Figure 2. Biodistribution of ^{99m}Tc-P2045 in athymic nude mice inoculated s.c. with 1 x 10^7 MDA-MB-468 cells mixed 1:1 with matrigel. Twenty one days after tumor cell inoculation, the mice were injected i.v. with 1 x 10^9 pfu of AdSSTr2 or a control adenovirus followed by i.v. injection ^{99m}Tc-P2045 two days later. Mice were sacrificed 4 h later and blood, lungs, liver, kidney, muscle, pancreas, and tumor were harvested, weighed and counted in a gamma counter. The data are represented as the % injected dose (ID) per gram of tissue. Each bar represents the mean tissue concentration ± standard deviation from a group of 3-5 animals .

Figure 1

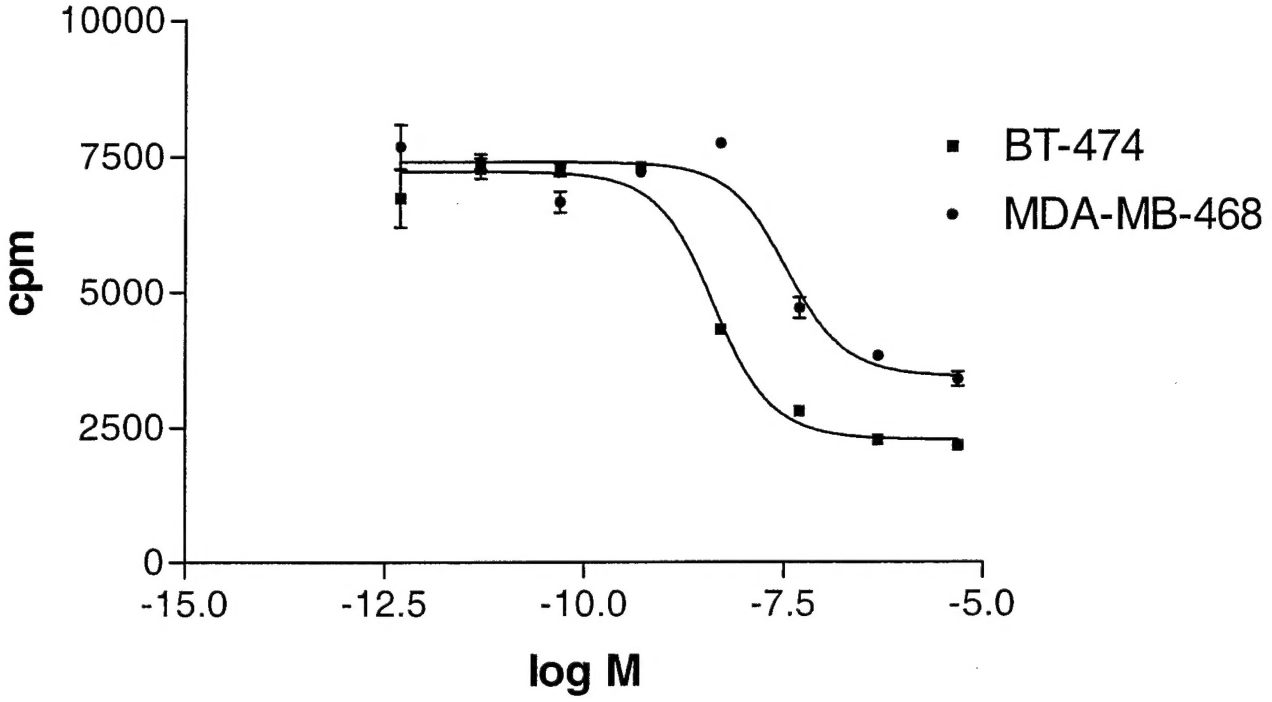
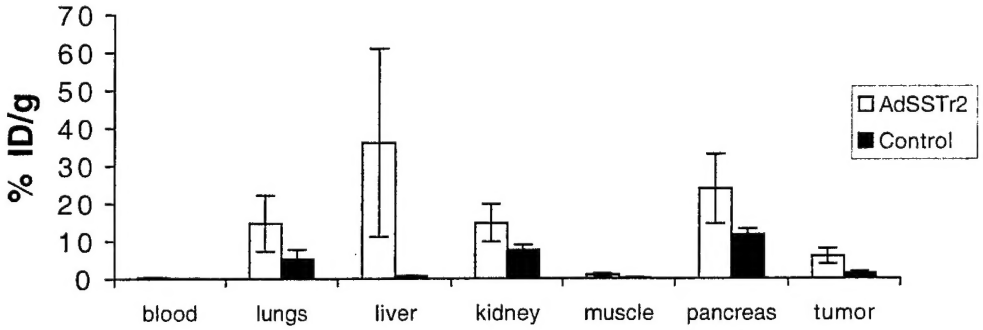

10000
7500
5000
2500
0
cpm
-15.0
-12.5
-10.0
-7.5
-5.0
log M
BT-474
MDA-MB-468

Table 1

	K_d (nM)	B_{max} (fmol/mg)
BT-474	0.8 ± 0.4	6,700 ± 200
MDA-MB-468	16.2 ± 16.1	99,600 ± 77,000

Figure 2


70
60
50
40
30
20
10
0
% ID/g
AdSSTr2
Control
blood
lungs
liver
kidney
muscle
pancreas
tumor